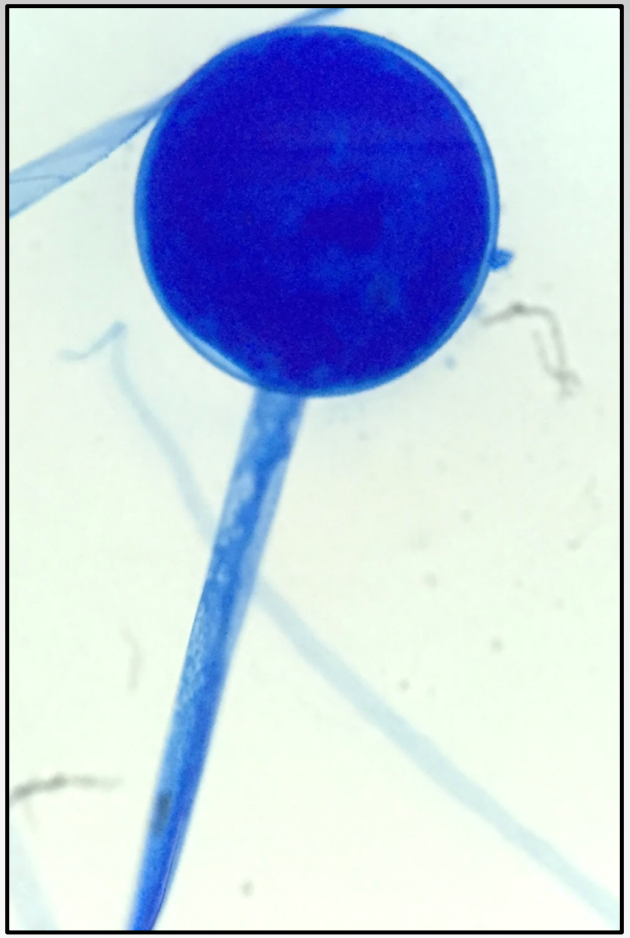
Induction of fungal biofilm in species known to cause Mucormycosis and its application to the antifungal activity of Amphotericin B and Thyme oil.

Karaleen Anderson, Mariel Hatch, Olga Kopp

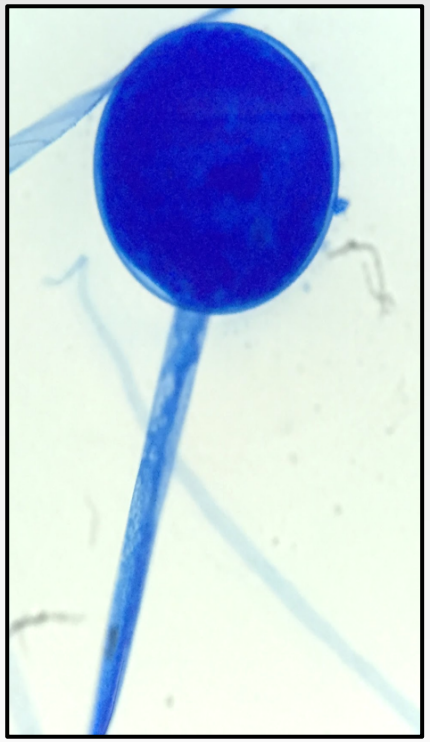
Introduction



Mucormycosis

- The unifying term used to describe infections caused by fungi belonging to the order Mucorales

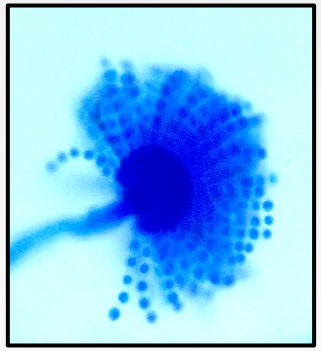
Species



Rhizopus oryzae



Absidia Corymbefiera



Rhizomucor pussillus

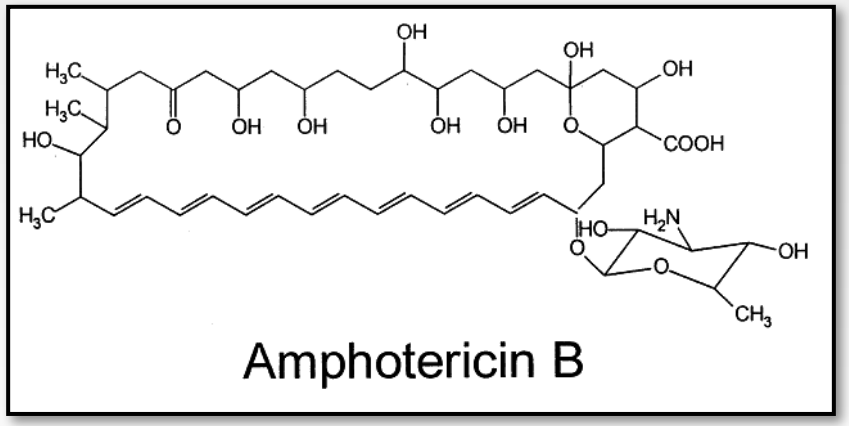
Opportunistic, progressive, necrotic & generally fatal

- Immunocompromised
- Blood disorders
- Corticosteroid use
- Diabetes
- Organ transplant recipient
- Burn patients

Current Treatment & Outcomes

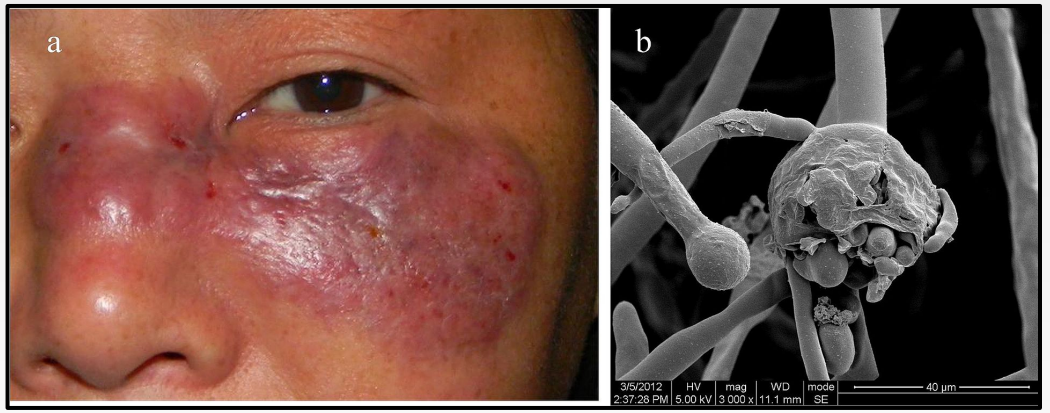
Treatment

- Surgical Debridement
- Amputation
- Amphotericin B
- Posacanazole



Outcomes: Vary depending on site of infection and strain of fungi.

- Overall mortality: 54%
- Sinus infection: 46%
- Pulmonary infection: 76%
- Disseminated: 97%

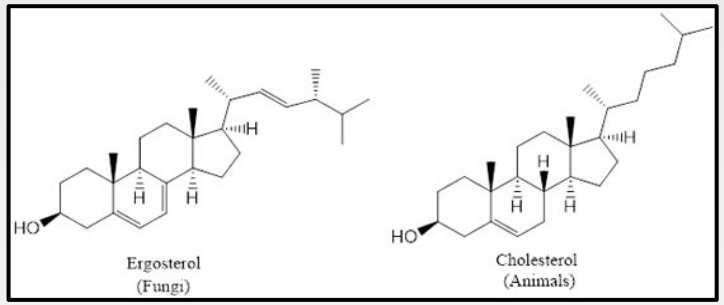


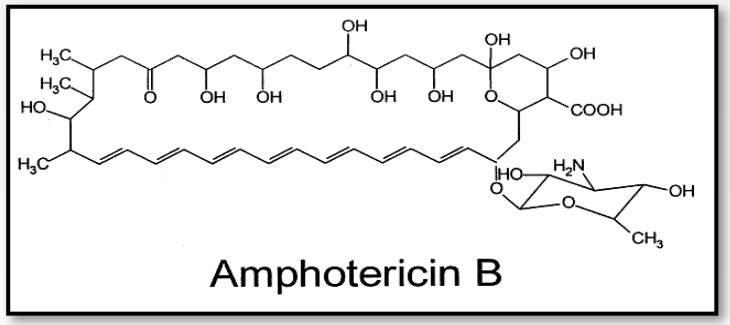
Ran Yuping (2016). "Observation of Fungi, Bacteria, and Parasites in Clinical Skin Samples Using Scanning Electron Microscopy". In Janecek, Milos; Kral, Robert. Modern Electron Microscopy in Physical and Life Sciences. InTech. doi:10.5772/61850. ISBN 978-953-51-2252-4.

Mechanism of Action

Amphotericin b

Exploits the difference in lipid composition of fungal (ergosterol) and mammalian cell membranes (cholesterol).





Side effects of Amphotericin B:

- Chills
- Fever
- Headache
- Loss of appetite
- Muscle or joint pain
- Stomach pain
- Weight loss
- Hepatotoxicity (Fatal)
- Nephrotoxicity (Fatal)

Combination Therapy & Essential oils

Combination:

- Reed et al (2008) noted that patients who received combination amphotericin B-caspofungin therapy had significantly better treatment success and survival time compared with patients who received amphotericin B alone.

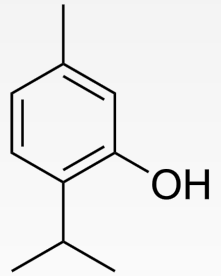
- Mota et al (2012) reported a highly synergistic effect when thyme and cinnamon were used in combination with amphotericin B compared with amphotericin B alone.

Essential Oils

Also interact with the fungal cell membrane and alter permeability.

Antifungal activity of an oil increases with the percentage of phenolic compounds found within the oil.

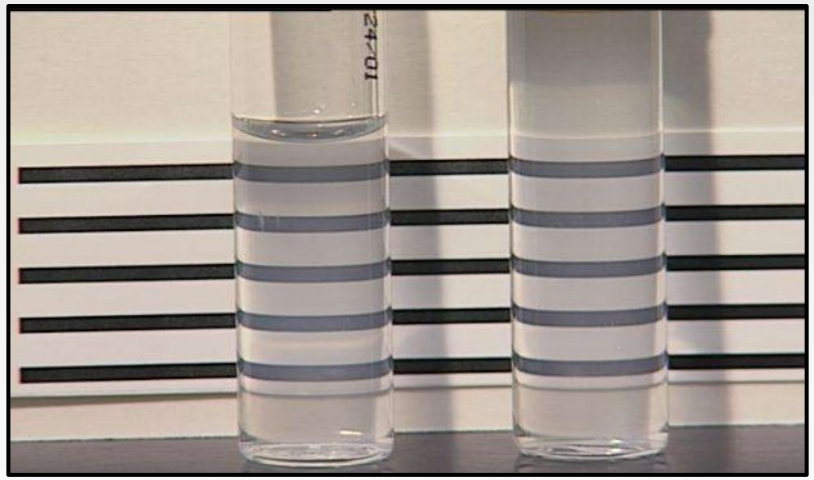
Thymol: Major phytochemical constituent found in Thyme: 46.6%



Planktonic vs Biofilm

Planktonic: Populations that grow in the test tube and flask cultures in the microbiology laboratory. Rarely found like this in nature.

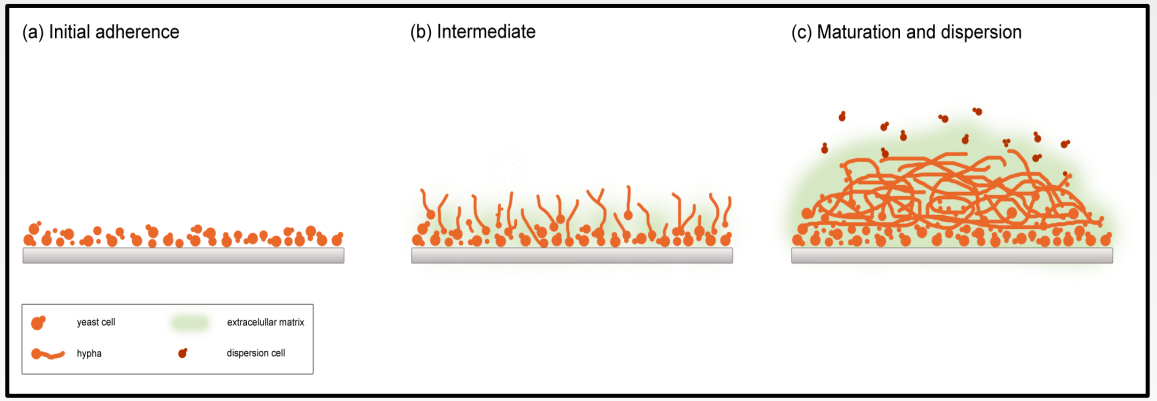
Example: McFarland Standard



https://microbeonline.com/preparation-mcfarland-turbidity-standards

Biofilm: Highly organized, surface associated communities of microorganisms that are protected within an extracellular matrix (Harding et al., 2009).

Once formed can have increased resistance to antifungal drugs.



https://www.intechopen.com/books/fungal-pathogenicity/biofilm-formation-as-a-pathogenicity-factor-of-medically-important-fungi

Hypothesis

The phytochemical constituents found in oil from *Thymus vulgaris* can be used to lower the minimum inhibitory concentration of amphotericin B used to treat Mucormycosis.

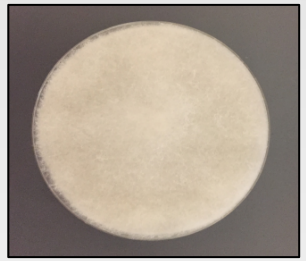


Methods



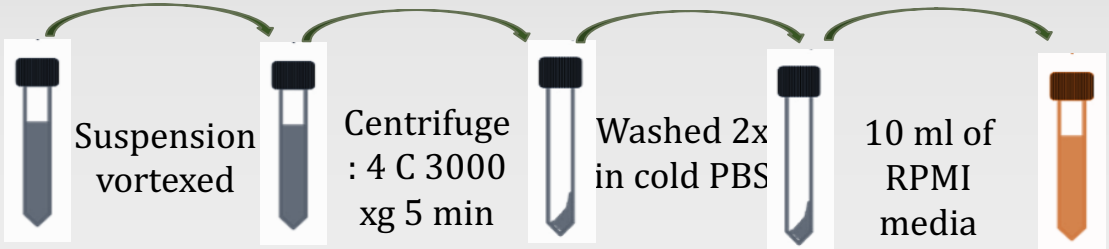
Procedure

Colonies grown on SDA for 3-5 days



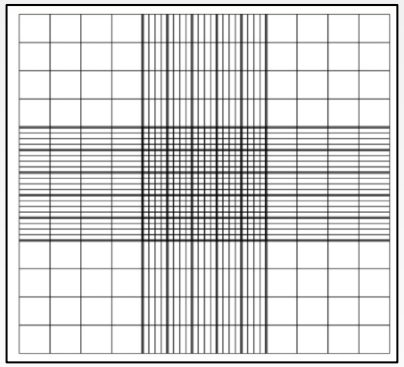
5 ml PBS/tween added to colonies

Suspension transferred to 10 ml sterile tube



**Vortex & Centrifuge between each wash

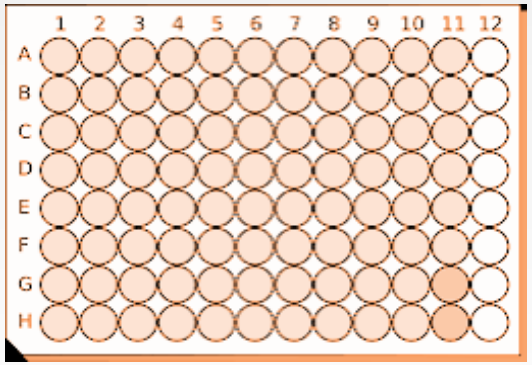
Hemocytometer cell counter



Solution diluted to cell density: 10^5 cells/ml

100 µl of suspension to each well

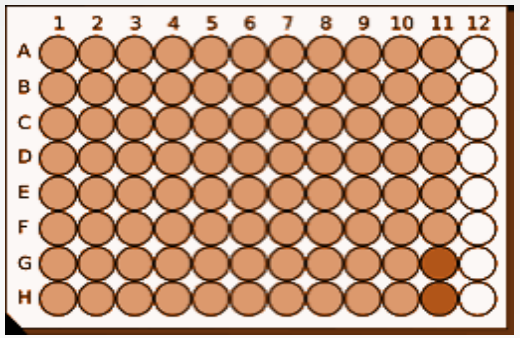
Incubated for 24 hours



200 µl XTT/menadione

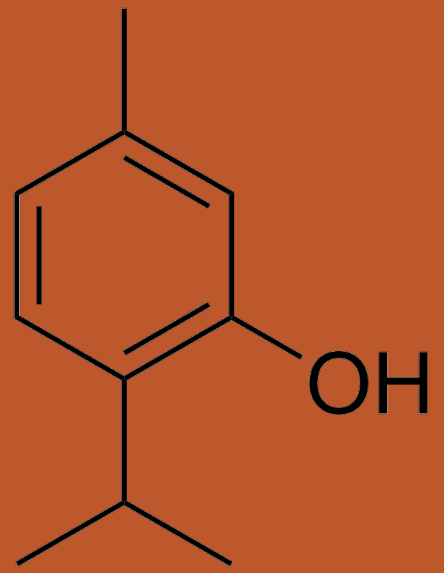
Biofilms aspirated and washed

Verification of equivalent biofilms



XTT reduced to formazan

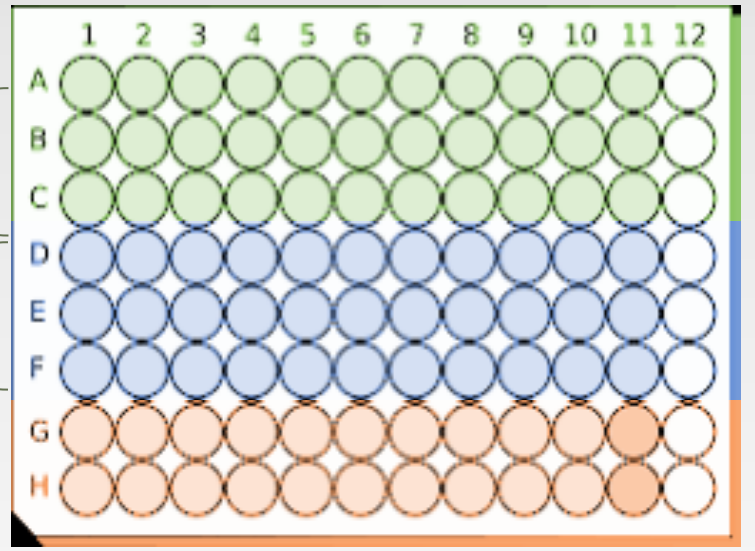
Antifungal Testing



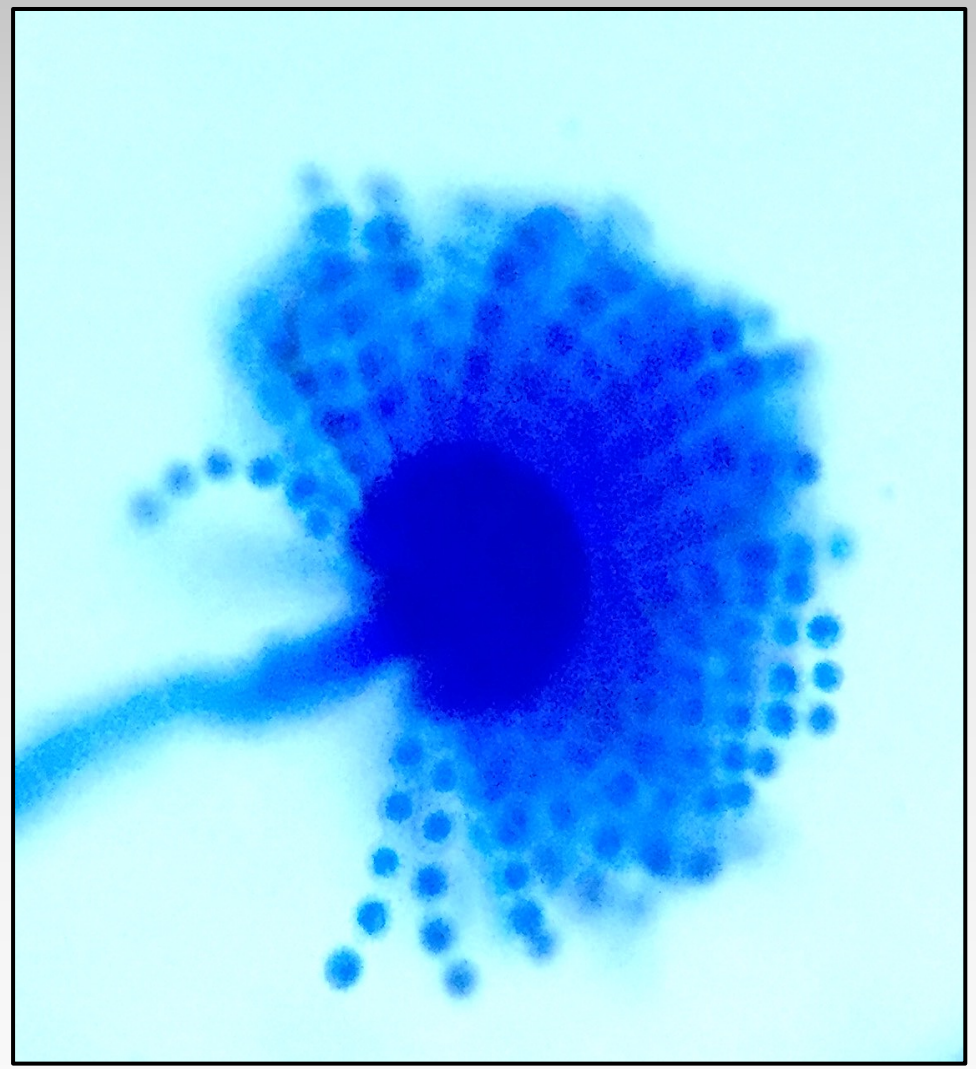
Response of biofilms to Thyme and Amp B

Thyme dilution

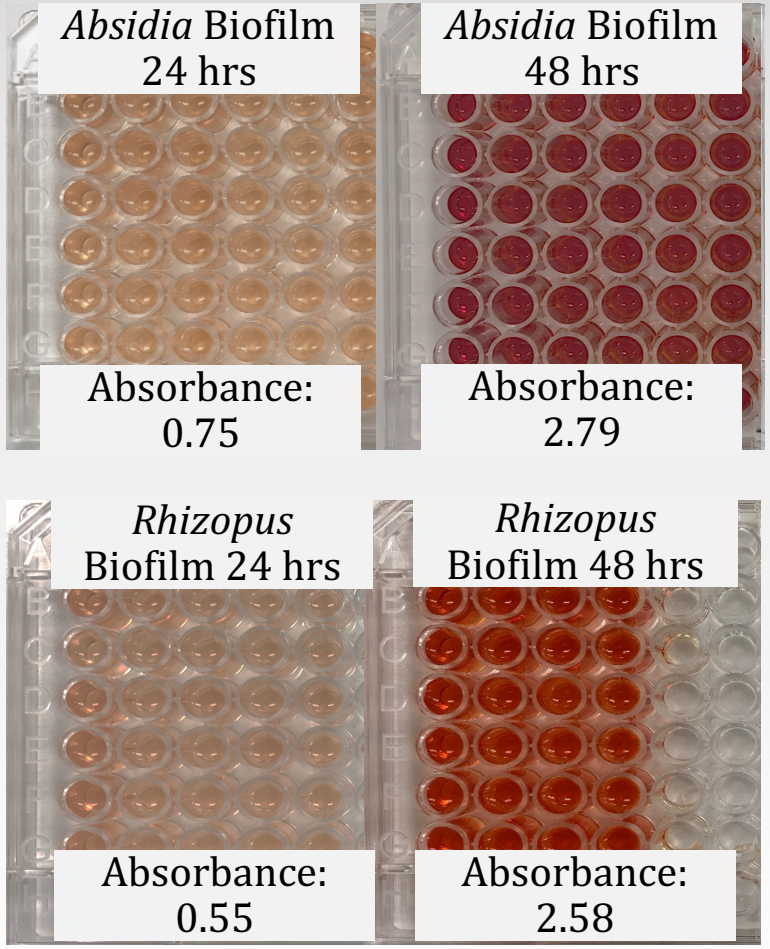
Amp B. dilution



Results



Comparison of incubation periods of 24 and 48 hours



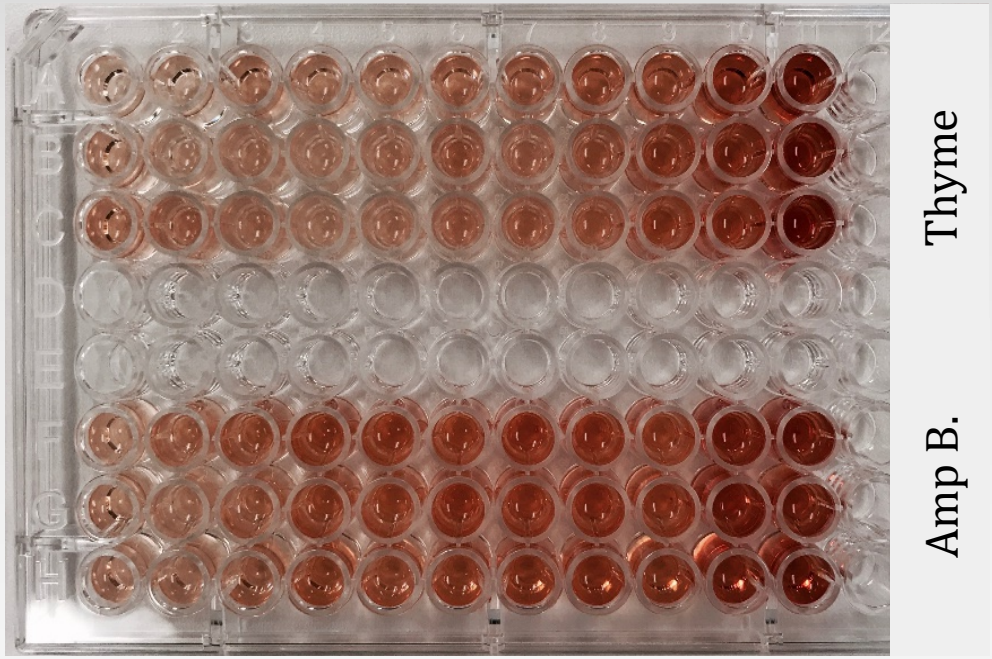
Results indicate that *Absidia corymbifera* and *Rhizopus oryzae* biofilms should be incubated for a total of 48 hours.

We suggest washing and re-suspending the biofilms after 24 hours in order to get optimal biofilm growth with this species.

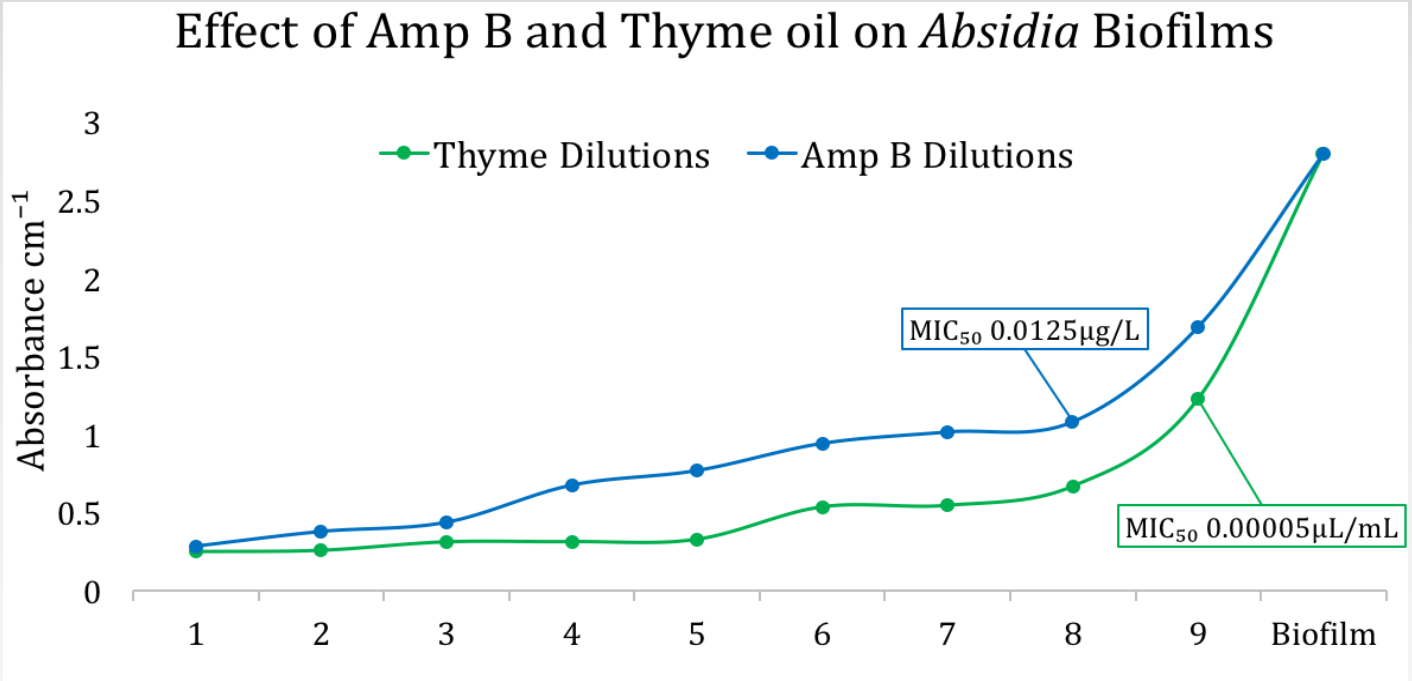
When washing and aspirating the biofilms, extra care should be taken when inverting the plates to avoid damaging the biofilm.

Rhizopus oryzae biofilms are particularly delicate and require extra care when washing and aspirating.

Antifungal treatment of *Absidia corymbifera* biofilms

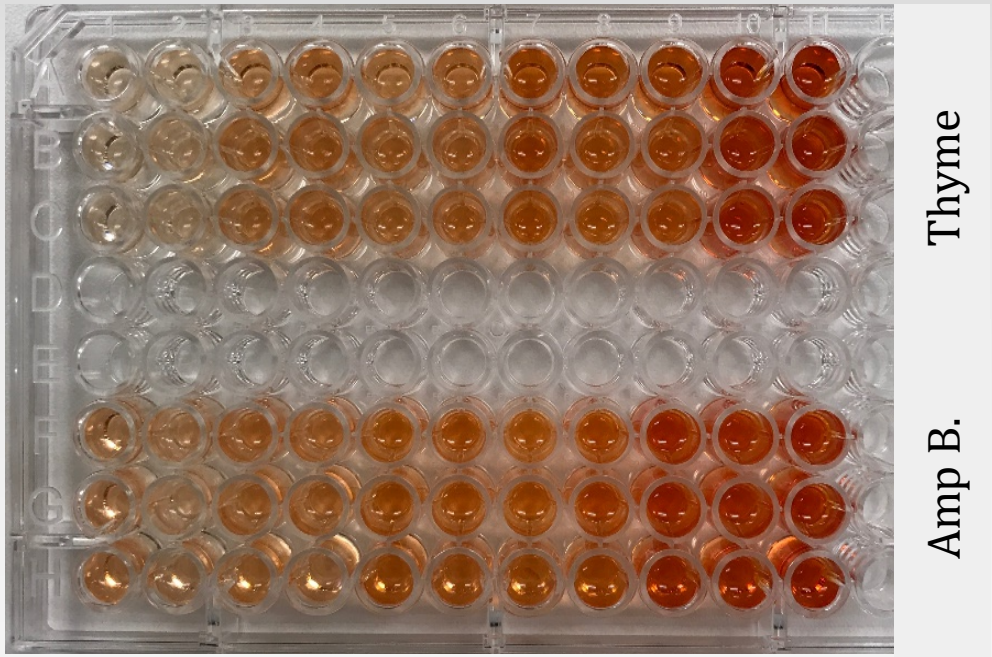


Absidia fungal biofilms treated with Thyme oil and Amp B showing a dose response gradient based on the concentration of each antifungal agent

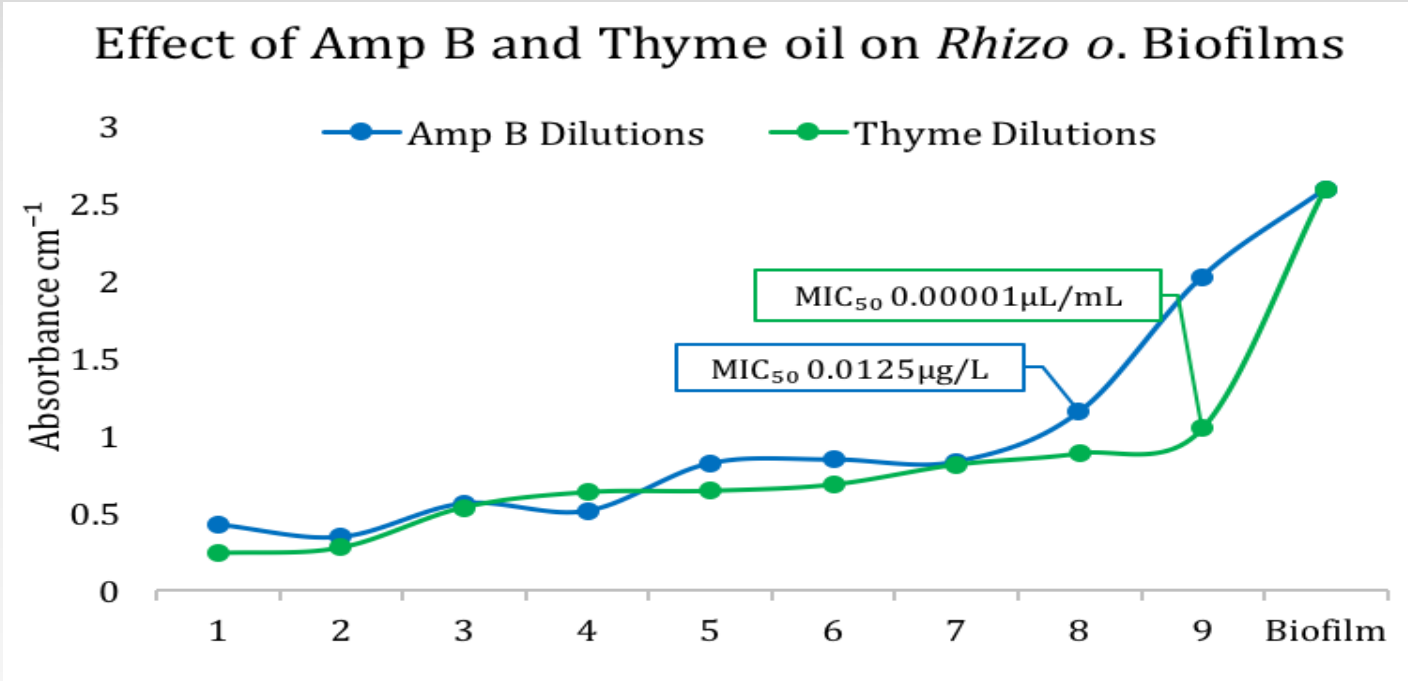


Absorbance vs Dilution in *Absidia.* The MIC_{50} found here will be compared to the MIC_{50} found in the synergism test to determine if Thyme is able to lower the concentration of Amp B needed to inhibit the biofilm.

Antifungal treatment of *Rhizopus oryzae* biofilms



Rhizopus o. fungal biofilms treated with Thyme oil and Amp B showing a dose response gradient based on the concentration of each antifungal agent



Absorbance vs Dilution in *Rhizopus o*. The MIC_{50} found here will be compared to the MIC_{50} found in the synergism test to determine if Thyme is able to lower the concentration of Amp B needed to inhibit the biofilm.

Synergistic Treatment

Synergistic effect of Amp B and thyme oil on biofilms

Thyme & Amp B. Synergism test

Horizontal Thyme dilution

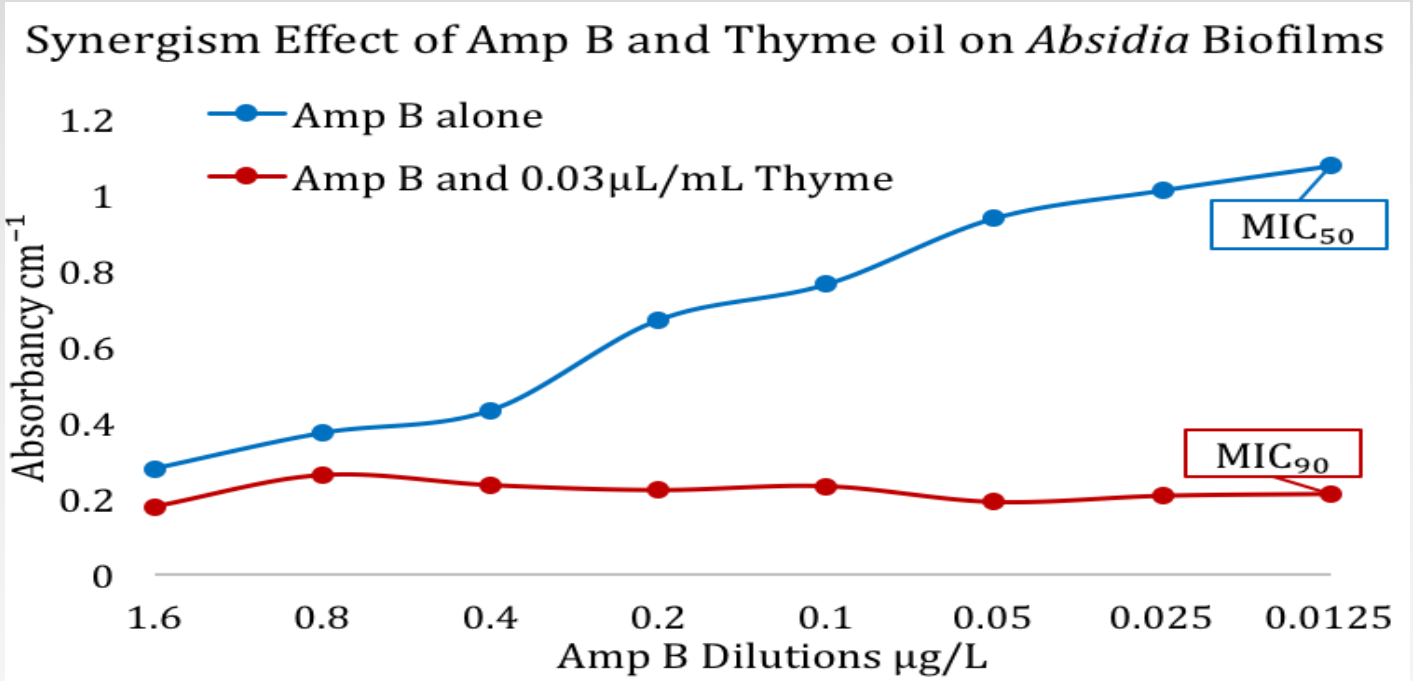
	1	2	3	4	5	6	7	8	9	10	11	12
A	O1A1	O2A1	O3A1	O4A1	O5A1	O6A1	O7A1	O8A1	O9A1	O10A1	+	-
B	O1A2	O2A2	O3A2	O4A2	O5A2	O6A2	O7A2	O8A2	O9A2	O10A2	+	-
C	O1A3	O2A3	O3A3	O4A3	O5A3	O6A3	O7A3	O8A3	O9A3	O10A3	+	-
D	O1A4	O2A4	O3A4	O4A4	O5A4	O6A4	O7A4	O8A4	O9A4	O10A4	+	-
E	O1A5	O2A5	O3A5	O4A5	O5A5	O6A5	O7A5	O8A5	O9A5	O10A5	+	-
F	O1A6	O2A6	O3A6	O4A6	O5A6	O6A6	O7A6	O8A6	O9A6	O10A6	+	-
G	O1A7	O2A7	O3A7	O4A7	O5A7	O6A7	O7A7	O8A7	O9A7	O10A7	+	-
H	O1A8	O2A8	O3A8	O4A8	O5A8	O6A8	O7A8	O8A8	O9A8	O10A8	+	-

Vertical Amp B dilution

Synergistic antifungal treatment of *Absidia c.* biofilms

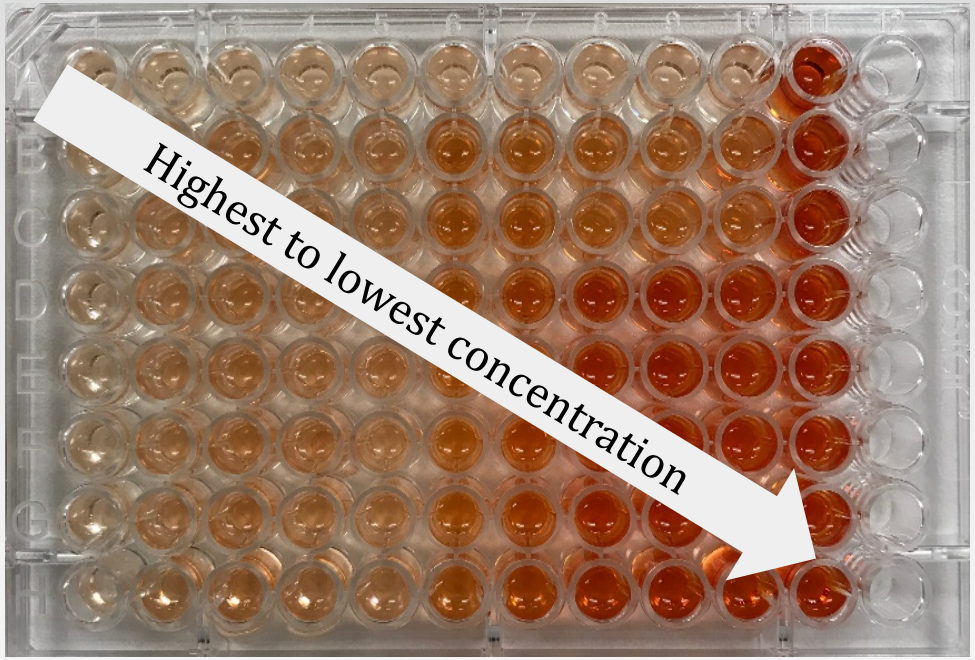


Synergism plate indicating that the higher concentration of Amp B and Thyme inhibited the biofilm to a greater degree.

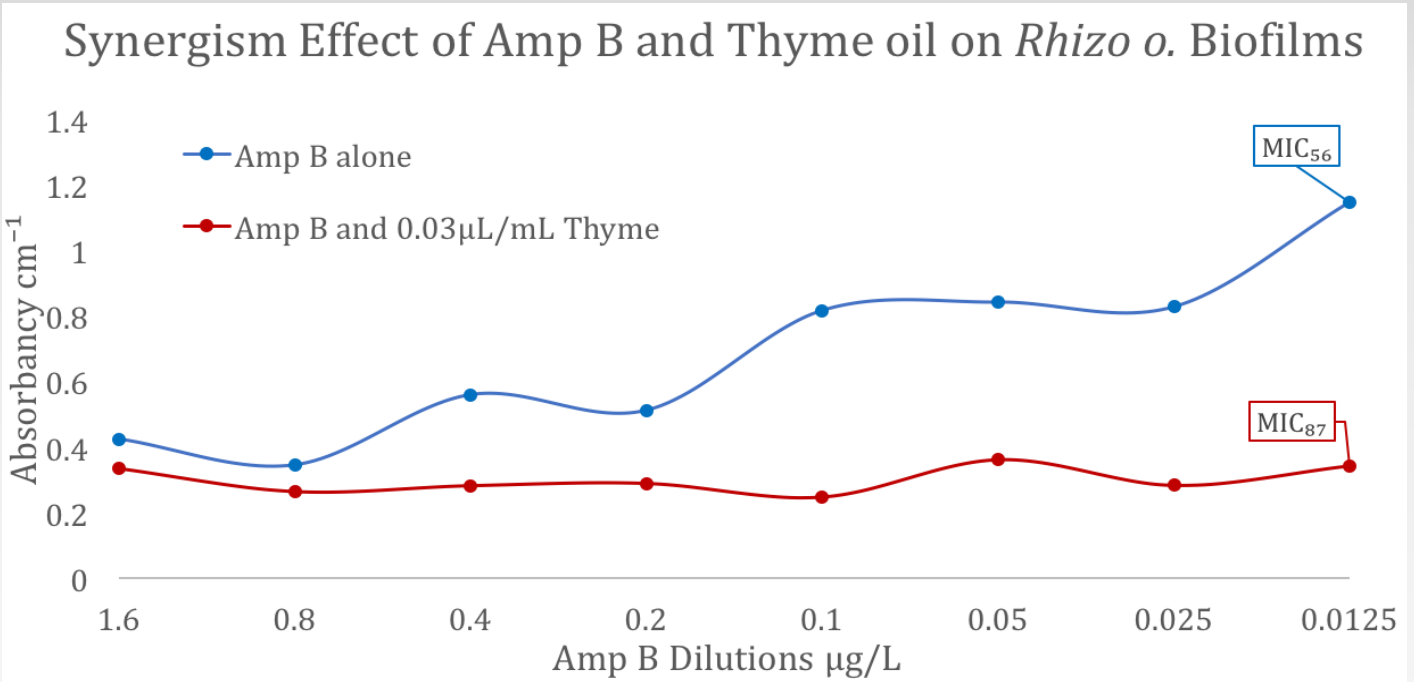


Synergism between Amp B and Thyme oil on Absidia biofilms show that the combination of Amp B and Thyme increases fungal inhibition compared to Amp B alone.

Synergistic antifungal treatment of *Rhizopus o.* biofilms



Synergism plate indicating that the higher concentration of Amp B and Thyme inhibited the biofilm to a greater degree.



Synergism graph showing curves for the highest thyme concentrations along the Amp B dilution gradient. Results show that the combination of Amp B and Thyme increases the amount of inhibition compared to Amp B alone.

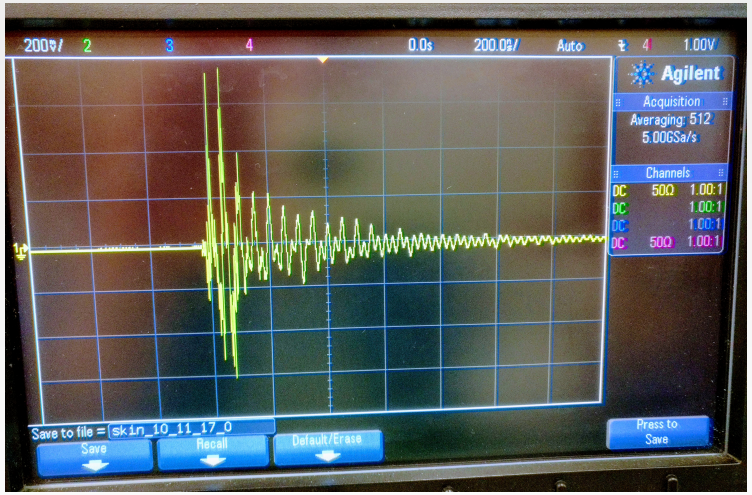
Conclusions and Discussion



Conclusions

- Capable of growing Rhizopus o. and Absidia c. fungal biofilm under laboratory conditions
- Antifungal properties of *Thymus vulgaris* still effective in treating *Rhizo O. and Absidia c.* fungi in biofilm morphology
- Thyme oil and Amphotericin B show synergistic properties in the treatment of *Rhizopus O. and Absidia c.* biofilm

Next Steps



- Testing 4 other oils that demonstrate antifungal properties as potential Mucormycosis treatments, namely:
  - Clove
  - Lemongrass
  - Oregano
  - Cinnamon

- Adding high frequency ultrasound treatment to oil and amp B treatment of biofilms to determine most successful combination of synergistic treatment of *Rhozopus O. and Absidia c.*

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